Advances in Brief

Prevention of Intestinal Toxic Effects and Intensification of Irinotecan’s Therapeutic Efficacy against Murine Colon Cancer Liver Metastases by Oral Administration of the Lipopeptide JBT 3002

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Abstract

The induction of severe diarrhea limits the usefulness of the DNA topoisomerase I inhibitor irinotecan (CPT-11) in the treatment of advanced colon cancer. We investigated whether oral administration of the new synthetic bacterial lipopeptide, JBT 3002, encapsulated in phospholipid liposomes could prevent damage to the intestinal epithelium and lamina propria and thus allow for the parenteral administration of high-dose irinotecan to mice with established syngeneic CT-26 colon cancer liver metastases. Treatment of mice with four daily i.p. injections of 100 mg/kg irinotecan was effective against liver metastases but also resulted in loss of body weight and early death. Histopathological examination of the intestines after this treatment revealed loss of villi, epithelial vacuolation, decrease in the number of cells in the crypts in S-phase, increase in the number of apoptotic cells, and reduction in the number of lymphocytes in the lamina propria. In contrast, treatment of mice with the same irinotecan regimen after oral administration of JBT 3002 produced highly significant inhibition of liver metastases without detectable damage to the intestines. Studies that used irinotecan administered once a week for 3 weeks after pretreatment with oral JBT 3002 demonstrated significantly intensified eradication of established CT-26 liver metastases compared with treatment with once-weekly irinotecan alone.

Histological studies revealed that the liver metastases in mice treated with oral JBT 3002 and i.p. irinotecan contained a higher number of macrophages than metastases in mice treated with either drug alone. In vitro studies revealed that irinotecan produced direct antiproliferative effects but JBT 3002 did not. Tumor cells exposed to both irinotecan and macrophages activated by JBT 3002 were highly susceptible to lysis. These data show that oral administration of JBT 3002 can prevent irinotecan-induced gastrointestinal toxic effects and maintain the integrity of the lamina propria, thus allowing for intensification of irinotecan therapy against liver metastases from colon cancer.

Introduction

Irinotecan (CPT-11), a semisynthetic derivative of camptothecin, is a potent chemotherapeutic agent gaining increased use in drug-refractory colorectal cancer (1-4). Irinotecan exerts S-phase-specific cytotoxicity through the inhibition of DNA topoisomerase I (5, 6). One of the major dose-limiting side effects of irinotecan is severe delayed-onset diarrhea accompanied by dehydration and electrolyte imbalance (1, 4). These symptoms are often treated with loperamide or diphenoxylate derivatives (7, 8), but side effects remain common.

Although the exact mechanism of irinotecan-induced diarrhea is unknown, a recent study in mice ascribed the toxicity to structural changes in intestinal architecture resulting from disordered epithelial cell differentiation and apoptosis rather than a secretory mechanism (9). Mucosal injury could also be a result of infection due to chemotherapy-induced depletion or dysfunction of leukocytes within the lamina propria (10) or lack of production of growth factors and cytokines by intestinal epithelial cells and cells of the lamina propria (11, 12).

Mucosal integrity and immunity may be preserved by macrophage-derived cytokines that regulate development and differentiation of lymphocytes within the lamina propria (13-17). Previous studies from our laboratory have demonstrated that the incubation of monocytes-macrophages with synthetic analogues of bacterial cell walls, such as MTP-PE,3 can induce production of monocyte-derived cytokines in vitro (18). Systemic administration of MLVs containing MTP-PE prevents destruction of the intestinal mucosa and monocytopenia induced by high doses of irinotecan (19). These data suggest that JBT 3002 might have similar protective effects.

The abbreviations used are: MTP-PE, muramyl tripeptide phosphatidylethanolamine; MLV, multilamellar liposome vesicle; IL, interleukin; BrdUrd, bromodeoxyuridine; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; PEM, peritoneal exudate macrophage; Scav-R, scavenger receptor; rIFN-γ, recombinant interferon γ.

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by chemotherapy with doxorubicin (19). Moreover, these immunomodulators activate tumoricidal properties in macrophages (18, 20, 21), which results in the eradication of lymph node, lung, and liver metastases in murine tumor models (22).

We have recently reported (23, 24) that a new synthetic lipopeptide (N-acetylated derivative of Ψ-amino-C1-C3-alkanesulfonic acid, JBT 3002) potently activates murine macrophages to become tumoricidal and to release IL-1α, IL-6, tumor necrosis factor-α, and nitric oxide. In the study reported here, we evaluated the ability of JBT 3002 to protect mice from the intestinal toxic effects produced by the administration of irinotecan for treatment of liver metastasis from syngeneic colon carcinoma. We found that the oral administration of JBT 3002 prevents damage to the intestines and enhances the therapeutic efficacy of irinotecan.

Materials and Methods

Animals. Specific pathogen-free BALB/c mice were purchased from the Animal Production Area of the National Cancer Institute (Frederick, MD). Animals were maintained according to institutional guidelines in facilities approved by the American Association for Accreditation of Laboratory Animal Care in accordance with United States Department of Agriculture, Department of Health and Human Services, and NIH regulations and standards.

Reagents. Eagle’s MEM, Ca²⁺- and Mg²⁺-free HBSS, and fetal bovine serum were purchased from M. A. Bioproducts (Walkersville, MD). JBT 3002 was obtained from Jenner Biotherapies (San Ramon, CA). 1-Palmitoyl-2-oleoyl-phosphatidylcholine and dioleoyl-phosphatidylserine were purchased from Avanti Polar Lipids (Birmingham, AL). Irinotecan, produced by Avanti Polar Lipids (Birmingham, AL) in a humidified atmosphere containing 5% CO₂ in air. All reagents used in tissue culture were free of endotoxin as determined by the Limulus amebocyte lysate assay (Associates of Cape Cod, Inc., Falmouth, MA).

Tumor Cell Cultures. CT-26 murine colon carcinoma cells syngeneic to BALB/c mice (25) were grown as monolayer cultures in MEM supplemented with 5% fetal bovine serum, vitamins, sodium pyruvate, l-glutamine, and nonessential amino acids. The adherent monolayer cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂ in air. All cultures were free of mycoplasma, reovirus type 3, pneumonia virus of mice, K virus, encephalitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (assayed by M. A. Bioproducts).

Preparation and Administration of Liposomes. 1-Palmitoyl-2-oleoyl-phosphatidylcholine and dioleoyl-phosphatidylserine (7:3 molar ratio) with or without the indicated amounts of JBT 3002 were dissolved in chloroform. MLVs were prepared by hydration of the lipid film with HBSS, followed by vigorous shaking for 6 min using a vortex shaker. Oral administration of JBT 3002 was performed using a rigid, curved feeding tube. Each dose consisted of 5 μmol of MLV suspended in 0.2 ml HBSS. For in vitro assay, the liposomes were diluted in medium before use.

Experimental Liver Metastasis. To prepare tumor cells for inoculation, CT-26 cells in exponential growth phase were harvested by a brief exposure to a solution of 0.25% trypsin and 0.1% EDTA. The cell suspension was pipetted to produce a single-cell suspension, washed, and resuspended in HBSS. Cell viability was determined by trypan blue exclusion, and only single-cell suspensions of >90% viability were used. Tumor cells (1 × 10⁶/0.05 ml HBSS) were injected into the spleens of BALB/c mice after laparotomy under methoxyflurane anesthesia. The incision was closed in one layer with wound clips (25). This protocol resulted in a 100% incidence of liver metastasis. The mice were euthanized when animals in the control group became moribund. Primary spleen tumor volume was estimated with the following formula: tumor volume = L (mm) × W² (mm²)/2, where L and W represent the length and the width of the tumor mass, respectively. The livers were harvested and placed in Bouin’s solution for 24 h before fixation in 10% buffered formalin. The number of experimental liver metastases was determined using a dissecting microscope. When the number of metastases exceeded 100, we assigned a value of >100. The median number of liver metastases was compared among the treatment groups using the Mann-Whitney U test.

Immunohistochemical Analysis. Mice were injected i.v. with 0.2 ml saline containing 250 μg BrdUrd 1 h before they were killed (26). The small and large intestines and the caudate lobe of the liver were resected, and tissue samples were fixed in 10% buffered formalin or snap-frozen in liquid nitrogen. Immunohistochemical staining was performed by the immunoperoxidase technique (27). Paraffin sections were dewaxed in xylene and then rinsed in graded ethanol hydration in PBS. Antigen retrieval was performed by incubating the sections with Pepsin Reagent (Biomeda Corp., Foster City, CA) for 20 min. For S-phase labeling with BrdUrd, the sections were incubated with 2 N HCl for 30 min at 37°C and then rinsed three times with PBS. Endogenous peroxidase was inactivated by incubating the sections with 3% hydrogen peroxide in methanol for 10 min. Nonspecific reactions were blocked by incubating the sections in a solution containing 5% normal horse serum and 1% normal goat serum. The sections were incubated with a 1:100 dilution of a monoclonal mouse antikeratin AE1/AE3 antibody that recognizes pan cytokeratin (Boehringer Mannheim Corp., Indianapolis, IN) or a 1:50 dilution of a monoclonal mouse anti-BrdUrd antibody (Becton Dickinson, Mountain View, CA) overnight at 4°C.

Frozen sections for staining with antimouse CD3 (specific for lymphocytes) or antimouse macrophage Scav-R were processed as described previously (27). The antibodies used were a monoclonal rat antimouse CD3 antibody (Sero-tec, Ltd., Oxford, England) at a dilution of 1:2000 and a monoclonal rat antimouse Scav-R antibody (Sero-tec) at a dilution of 1:70. Antibodies used for developing were goat antimouse IgG + IgM antibody (Jackson ImmunoResearch Labs., Inc., West Grove, PA) at a dilution of 1:200 for keratin, antimouse IgG1 antibody (PharMingen) at a dilution of 1:100 for BrdUrd, and antirat IgG antibody (Jackson ImmunoResearch) at a dilution of 1:200 for CD3 and Scav-R.
Table 1  Therapy of experimental liver metastases produced by murine CT-26 colon carcinoma with irinotecan with or without oral JBT 3002

BALB/c mice were injected in the spleen with $1 \times 10^6$ viable CT-26 cells on day 0. Mice were treated with oral feedings of 5 μmol MLV-HBSS or MLV-JBT 3002 (1 μg/dose) for 3 consecutive days beginning 3 days after tumor cell inoculation. Seven days later, groups of mice received 4 daily i.p. injections of irinotecan (CPT-11). Mice were killed on day 18–20.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ΔBW (%)</th>
<th>Incidence</th>
<th>Mean tumor volume (mm³)</th>
<th>Incidence</th>
<th>Median, n (range)</th>
<th>Liver weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLV-HBSS</td>
<td>6.6</td>
<td>16 of 16</td>
<td>$172 \pm 71$</td>
<td>16 of 16</td>
<td>50 (5-&gt;100)</td>
<td>2.8 ± 1.0</td>
</tr>
<tr>
<td>MLV-HBSS + CPT-11 (50 mg/kg)</td>
<td>3.6</td>
<td>13 of 13</td>
<td>$125 \pm 37^b$</td>
<td>9 of 13</td>
<td>10 (0-&gt;100)</td>
<td>2.0 ± 0.8^b</td>
</tr>
<tr>
<td>MLV-HBSS + CPT-11 (100 mg/kg)</td>
<td>-22.1</td>
<td>15 of 15</td>
<td>$48 \pm 20^c$</td>
<td>1 of 15</td>
<td>0 (0-5)^c</td>
<td>1.5 ± 0.3^c</td>
</tr>
<tr>
<td>MLV-JBT 3002</td>
<td>8.2</td>
<td>15 of 15</td>
<td>$194 \pm 68$</td>
<td>15 of 15</td>
<td>20 (5-&gt;100)</td>
<td>2.5 ± 1.1</td>
</tr>
<tr>
<td>MLV-JBT 3002 + CPT-11 (100 mg/kg)</td>
<td>-6.1</td>
<td>13 of 15</td>
<td>$20 \pm 8^d$</td>
<td>1 of 15</td>
<td>0 (0-5)^d</td>
<td>1.4 ± 0.1^d</td>
</tr>
</tbody>
</table>

*ΔBW, changes in body weight, calculated by the formula $ΔBW (%) = (A - B)/B \times 100$, where $A$ is the mean body weight of mice at death and $B$ is the mean body weight of mice on day 0.

*P < 0.05 as compared with MLV-HBSS.

*Six mice died during therapy. They were free of liver metastasis.

*P < 0.005 as compared with MLV-HBSS.

Table 2  Therapy of experimental liver metastases produced by murine CT-26 colon carcinoma with intensive irinotecan therapy in combination with oral JBT 3002 given on different schedules

BALB/c mice were injected in the spleen with $1 \times 10^6$ viable CT-26 cells on day 0. Mice were treated with oral feedings of 5 μmol MLV-HBSS or MLV-JBT 3002 (1 μg/dose) for 3 consecutive days beginning 3 days after tumor cell inoculation. Seven days later, groups of mice received 4 daily i.p. injections of 100 mg/kg irinotecan (CPT-11). All groups were killed on day 18.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ΔBW (%)</th>
<th>Incidence</th>
<th>Mean tumor volume (mm³)</th>
<th>Incidence</th>
<th>Median, n (range)</th>
<th>Liver weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLV-HBSS</td>
<td>4.5</td>
<td>5 of 5</td>
<td>$147 \pm 62$</td>
<td>5 of 5</td>
<td>44 (7-&gt;100)</td>
<td>2.4 ± 0.8</td>
</tr>
<tr>
<td>MLV-HBSS + CPT-11</td>
<td>-18.3</td>
<td>5 of 5</td>
<td>$44 \pm 18^b$</td>
<td>1 of 5^c</td>
<td>0 (0-1)^b</td>
<td>1.5 ± 0.2^b</td>
</tr>
<tr>
<td>MLV-JBT 3002 (3 days)</td>
<td>8.9</td>
<td>5 of 5</td>
<td>$185 \pm 58$</td>
<td>5 of 5</td>
<td>21 (3-&gt;100)</td>
<td>2.2 ± 0.8</td>
</tr>
<tr>
<td>MLV-JBT 3002 (3 days) + CPT-11</td>
<td>-4.0</td>
<td>5 of 5</td>
<td>$19 \pm 7^d$</td>
<td>0 of 5</td>
<td>0 (all 0)^d</td>
<td>1.5 ± 0.3^d</td>
</tr>
<tr>
<td>MLV-JBT 3002 (2 days) + CPT-11</td>
<td>-2.2</td>
<td>4 of 5</td>
<td>$18 \pm 12^e$</td>
<td>0 of 5</td>
<td>0 (all 0)^e</td>
<td>1.4 ± 0.1^e</td>
</tr>
<tr>
<td>MLV-JBT 3002 (1 day) + CPT-11</td>
<td>-3.0</td>
<td>5 of 5</td>
<td>$17 \pm 5^d$</td>
<td>1 of 5</td>
<td>0 (0-2)^d</td>
<td>1.4 ± 0.1^d</td>
</tr>
</tbody>
</table>

*ΔBW, changes in body weight, calculated by the formula $ΔBW (%) = (A - B)/B \times 100$, where $A$ is the mean body weight of mice at death and $B$ is the mean body weight of mice on day 0.

*P < 0.05 as compared with MLV-HBSS.

*Two mice died during therapy. They were free of liver metastasis.

*P < 0.005 as compared with MLV-HBSS.

*P < 0.005 as compared with MLV-HBSS + CPT-11.

After three rinses with physiological saline, the sections were incubated with dianinobenzidine substrate (Research Genetics, Huntsville, AL) for 5 min. The sections were rinsed with distilled water and counterstained with Mayer’s hematoxylin.

Apoptotic cells in intestinal tissues were detected by TUNEL assay exactly as previously described (28). BrdUrd- and TUNEL-positive cells in the intestines and Scav-R-labeled cells in the liver were counted under a light microscope at ×200 (i.e., objective ×20 and ocular ×10). The results are presented as the number of positive cells/0.25 mm² for BrdUrd and TUNEL and the number of positive cells/0.01 mm² for Scav-R.

Isolation and Activation of Macrophages.  PEMS were collected by peritoneal lavage of BALB/c mice given an i.p. injection of 1.5 ml of thioglycollate broth 4 days before being killed (23). The cells were washed with HBSS, and $1 \times 10^5$ cells that were resuspended in 0.1 ml serum-free MEM were plated into 96-well microculture plates. One h later, the nonadherent cells were removed by washing with medium. At that time, more than 98% of adherent cell populations were composed of macrophages according to morphological and phagocytic criteria (20). PEMS were then incubated for 20 h with medium alone or with medium containing rIFN-γ (10 units/ml) plus MLV-JBT 3002 (50 nmoi/well).  

In Vitro Cytotoxicity Assay. Macrophage-mediated tumor cytotoxicity was assessed by a radioactive release assay as described previously (20, 29). CT-26 cells in their exponential growth phase were incubated for 24 h in medium containing 0.2 μCi/ml [%H]thymidine (>2500 Ci/mmol; ICN Biomedicals, Costa Mesa, CA). The cells were washed three times with HBSS to remove unbound radioisotope, harvested by a brief trypsinization, and resuspended in medium. The target cells were plated (1 × 10⁴ cells/well) into wells containing control or test macrophages to obtain an initial macrophage:target cell ratio of 10:1. Radiolabeled target cells were also plated alone as a negative control for spontaneous macrophage activation. Three h later, irinotecan at different concentrations was added to each well. At 72 h after...
plating, the cultures were washed twice with physiological saline, and adherent viable cells were lysed with 0.1 ml of 0.1 n KOH. The lysates were harvested with a Harvester 96 (Tomtec, Orange, CT) and counted in a liquid scintillation counter. Percentage cytotoxicity was calculated with the following formula: cytotoxicity (%) = (A - B)/A × 100, where A is cpm in cultures of target cells alone (no irinotecan), and B is cpm in other test cultures.

Fig. 1 Histopathological changes in the ileum of BALB/c mice treated with MLV-HBSS (control), MLV-HBSS plus irinotecan (CPT-11), or MLV-JBT 3002 plus irinotecan (JBT 3002/CPT-11). BALB/c mice were injected in the spleen with 1 × 10⁶ viable CT-26 cells on day 0. Mice were treated with oral feedings of 5 μmol MLV-HBSS or 5 μmol MLV-JBT 3002 containing 1 μg JBT 3002/dose for 3 consecutive days beginning 3 days after tumor cell inoculation. Seven days later, groups of mice received 4 daily i.p. injections of CPT-11 (100 mg/kg). Mice were killed on day 18. Tissue sections from the ileum were analyzed for morphology with H&E staining, epithelial organization with antikeratin antibody, S-phase labeling with anti-BrdUrd antibody, apoptosis (TUNEL), lymphocytes in the lamina propria by staining with anti-mouse CD3 antibody, and for macrophage infiltration with antimouse macrophage Scav-R antibody. bar, 50 μm.
Statistical Analysis. The significance of the in vitro data was analyzed by unpaired Student’s t test. The significance of the in vivo data was analyzed by the Mann-Whitney U test and Student’s t test. Ps that were <0.05 were regarded as statistically significant.

Results

Therapeutic Effects of Irinotecan Administered Alone.

In the first set of experiments, we determined the dose of irinotecan necessary to inhibit experimental liver metastases. BALB/c mice were given intrasplenic injections of $1 \times 10^4$...
viable CT-26 cells on day 0. Seven days later, mice were treated with i.p. injections of 50 or 100 mg/kg irinotecan daily for 4 days. These doses of irinotecan were similar to those given in a previous study (9). Although these doses were higher than the maximum tolerated dose identified in clinical studies (125–350 mg/m²; Refs. 1–4), they did not produce immediate death in mice. As shown in Table 1, treatment with irinotecan at a dose of 50 mg/kg produced only a modest therapeutic effect, whereas the administration of 100 mg/kg resulted in significant regression of splenic tumors and complete inhibition of liver metastasis in 14 of 15 treated mice. However, severe diarrhea and corresponding loss of body weight were evident in all of the mice treated with 100 mg/kg irinotecan, and 6 of 15 treated mice died within 7 days after the last injection (Table 1).

**Theorpe tic Effects of Irinotecan Administered after Three Daily Oral Doses of JBT 3002.** Next, BALB/c mice were given intrasplenic injections of 1 x 10⁴ viable CT-26 cells. Three days later, the mice were given JBT 3002 by mouth. Because we have found previously (23) that JBT 3002 is significantly more potent than MTP-PE for activation of macrophages, we used a dose of 1 μg JBT 3002 contained in 5 μmol MLV. After three consecutive daily doses of JBT 3002, the mice were given i.p. injections of 100 mg/kg irinotecan once daily for 4 consecutive days. As shown in Table 1, all of the mice treated with this combination survived the high dose of irinotecan, and this dose significantly suppressed both primary spleen tumors and liver metastases (P < 0.005). No discernible differences in the incidence or median number of hepatic metastases were found between control mice or those receiving only oral JBT 3002.

**Effects of Intensive Irinotecan Therapy Administered after JBT 3002 Given on Different Schedules.** In the next set of experiments, we evaluated the effect of varying the schedule of JBT 3002 administration. Mice were given i.p. injections of 1 x 10⁴ viable CT-26 cells on day 0. Groups of five mice were given one, two, or three daily doses of 5 μmol MLV-HBSS or 1 μg JBT 3002 in 5 μmol MLV by mouth starting 3 days after tumor cell inoculation. Seven days after tumor cell injection, some mice were given four daily i.p. injections of 100 mg/kg irinotecan. The mice were euthanized and necropsied when the control group became moribund (on day 18 after tumor cell injection). The results of this second set of experiments are shown in Table 2. The size of the primary spleen tumors and the incidence and median number of experimental liver metastases were significantly reduced in the mice treated with irinotecan (P < 0.05). However, mice that were treated with irinotecan alone again had severe diarrhea associated with intestinal bleeding. Two of five mice died within 7 days after the last treatment, and three of five mice became moribund by day 18 of the study. Upon necropsy, we found that the small intestines of all mice in this group were shortened and had thickened walls. Notably, oral administration of JBT 3002 before i.p. irinotecan intensified the antitumor effects of irinotecan (P < 0.005). Moreover, this combination therapy was not associated with significant loss in body weight, and none of the mice in this group died.

Tissues from the small and large intestines of all mice were harvested and processed for histopathological analysis. H&E staining revealed severe morphological changes in the ileum (Fig. 1) and colon (Fig. 2) of mice injected with 100 mg/kg irinotecan. Loss of villi, epithelial vacuolation, and inflammatory cell infiltrates were found in the ileum; and hyperplasia, regeneration of glandular tubules, and scattered crypt abscesses were found in the colon. In contrast, in mice treated with JBT 3002 before irinotecan, the architecture of the ileum remained ordered (Fig. 1), and the colonic mucosa exhibited only mild goblet-cell hyperplasia (Fig. 2).

Epithelial cell organization and the presence of proliferating and apoptotic cells in the intestinal mucosa were determined by immunohistochemical staining for keratin (30), S-phase labeling with anti-BrdUrd antibody (26), and TUNEL (28), respectively (Fig. 1 and 2). This analysis revealed loss of tissue...
Stability that was associated with a nearly two-thirds reduction in the number of dividing cells in the crypts and a nearly 8-fold increase in the number of apoptotic cells along the mucosal surface (Fig. 3). These toxic effects were greatly diminished when mice were given one, two, or three oral doses of JBT 3002 before irinotecan.

We also characterized the presence of leukocytes within the lamina propria (Fig. 1 and 2). In control mice, the lamina propria was occupied by lymphocytes and macrophages as determined by labeling with CD3 and Scav-R, respectively. In contrast, in mice that received repeated i.p. injections of 100 mg/kg irinotecan, the structure of the lamina propria was thoroughly destroyed, and the population of CD3-positive cells was markedly decreased, whereas a large infiltrate of Scav-R-positive cells (macrophages) was observed. In mice that received oral JBT 3002 before irinotecan, the populations of these immune cells were normal (Fig. 1 and 2).

Effects of Once-Weekly Irinotecan Therapy Administered after JBT 3002 Given on Different Schedules. Since the intensive schedule of chemotherapy in the second set of experiments was too toxic to allow full evaluation of the efficacy of the combination therapy, we conducted a third set of experiments, in which we administered irinotecan on a protracted schedule of once a week for 3 weeks, similar to the schedule used to treat colon cancer in humans (2, 4). In these experiments, mice were given intrasplenic injections of $1 \times 10^4$ viable CT-26 cells. Three days later, groups of mice ($n = 5$) were given oral MLV-HBSS (control), or 1 $\mu$g JBT 3002 in 5 $\mu$mol phospholipid daily for 1, 2, or 3 days. On day 7 after tumor cell injection, some mice were given a single i.p. injection of 100 mg/kg irinotecan. This treatment schedule was repeated weekly for 3 weeks. The mice were killed and necropsied when the control group became moribund (day 24 after tumor cell injection). As shown in Table 3, once-per-week administration of irinotecan produced a significant reduction in the size of spleen tumors ($P < 0.05$) and the extent of liver metastases ($P < 0.005$), albeit to a lesser degree than found for the schedule of four daily injections of irinotecan. The combination of oral JBT-3002 and i.p. irinotecan produced a further reduction in the number of liver metastases (~4-fold) as compared with irinotecan alone ($P < 0.005$).

Macrophage Infiltration into Hepatic Metastases. Using immunohistochemical methods, we looked for changes in macrophage infiltration within the CT-26 experimental liver metastases. Our findings are shown in Fig. 4. In control mice, the majority of the cells that stained with the macrophage marker Scav-R were found on the periphery of the lesions; only a few infiltrated the metastases (mean, 11 ± 2 cells/0.01 mm²). In contrast, in mice treated with irinotecan, there was an intense infiltration of macrophages into the liver tumor nodules. The number of Scav-R-positive cells was 33 ± 4 cells/0.01 mm², a significant increase compared with the number in control mice ($P < 0.001$). In mice given JBT 3002 without irinotecan, the number of Scav-R-positive cells within metastases was not increased (mean, 14 ± 6 cells/0.01 mm²). However, mice treated with JBT 3002 before irinotecan had a dense inflammatory infiltrate (mean, 67 ± 10 cells/0.01 mm²) that actually obscured recognition of viable tumor cells. The immunohistochemical analyses of macrophage infiltration correlated with the macroscopic findings described in Table 3.

Mechanism of Effect of Combination Therapy with JBT 3002 and Irinotecan. We next examined a possible mechanism to explain the effects of the combination therapy against liver metastases. To rule out direct antiproliferative effects, we incubated CT-26 cells for 4 days with different concentrations of MLV-JBT 3002 (0–100 nmol/38-mm² well; 1 $\mu$g JBT 3002/300 $\mu$mol phospholipids) with or without different concentrations of irinotecan. Four days later, the number of viable tumor cells was determined. MLV-JBT 3002 did not alter the direct antiproliferative effects of irinotecan (IC$_{50}$, ~8 $\mu$g/ml; data not shown).

We then examined whether the activation of macrophages by MLV-JBT 3002 (23, 24) could contribute to the lysis of tumor cells by irinotecan. Results of a representative experiment are shown in Fig. 5. The peritoneal macrophages incubated with medium (control) were not cytotoxic against CT-26 cells. The
Fig. 4 Macrophage infiltration into CT-26 hepatic metastases. BALB/c mice were injected intrasplenicly with viable $1 \times 10^7$ viable CT-26 cells on day 0. The mice were treated with repeated oral feedings of 5 µmol MLV-HBSS or 5 µmol MLV containing 1 µg JBT 3002/ dose three times/week for 3 weeks beginning 3 days after tumor cell inoculation. Some mice received i.p. injections of 100 mg/kg CPT-11 once a week (on days 7, 14, and 21). All mice were killed on day 24 and necropsied. Liver sections were fixed in 10% buffered formalin for H&E staining or in liquid nitrogen for immunohistochemical staining with antimouse macrophage Scav-R antibody. bar, 50 µm.

exposure of tumor cells to 7 µg/ml and 10 µg/ml of irinotecan resulted in 24.5% and 33.5% cytotoxicity, respectively. The incubation of CT-26 target cells with macrophages activated with MLV-JBT 3002 plus rIFN-γ resulted in 50% cytotoxicity ($P < 0.05$), which increased to 65% ($P < 0.005$) in the presence of 10 µg/ml irinotecan.

Discussion
The present study demonstrates that oral administration of JBT 3002, a new synthetic lipopeptide and macrophage activator (23, 24) overcomes irinotecan-induced damage to intestinal epithelium and enhances the therapeutic efficacy of irinotecan against liver metastases of CT-26 murine colon carcinoma. Significant inhibition of liver metastases of murine colon cancer required repeated daily injections of 100 mg/kg irinotecan (CPT-11). Unfortunately, at this dose, all of the mice developed severe intestinal damage, and many died within 1 week after the fourth (final) i.p. injection. Treatment with 50 mg/kg of irinotecan was less toxic but also less effective. These data suggest that to have a significant therapeutic effect, irinotecan must be given at a relatively high dose that is likely to produce damage to the intestines. Oral administration of JBT 3002 before irino-
The development of severe diarrhea limits the clinical use of irinotecan (1, 2, 4). Topoisomerase I inhibitors such as irinotecan are highly S-phase specific; therefore, diarrhea may result from the direct toxic effects of irinotecan against dividing epithelial cells in the crypt of the intestines (6, 31) as well as from induction of apoptosis in the differentiated epithelium (9). Immunohistochemical analyses of the intestines showed a 3-fold decrease of S-phase labeling with BrdUrd and a 10-fold increase in the number of TUNEL-positive cells in mice given 100 mg/kg irinotecan daily for 4 days. In contrast, in mice that received repeated oral doses of the synthetic lipopeptide JBT 3002 before treatment with irinotecan, there was increased cell division in the crypts and a lower rate of cell death in the villi; and these were associated with a normalized epithelial organization. These findings indicate that JBT 3002 probably prevents chemotherapy-induced injury to the intestinal mucosa by elevating epithelial stem cell proliferation and reducing the incidence of apoptosis.

The present results confirm the findings that in mice, irinotecan-induced gastrointestinal toxicity was associated with structural changes in intestinal architecture resulting from disordered epithelial cell differentiation and apoptosis (9). Whether this pathology is also responsible for secretory diarrhea in humans is unclear.

Recent studies have demonstrated that a variety of growth factors and cytokines, produced both by the epithelium itself and by leukocytes within the lamina propria, may regulate the proliferation of intestinal epithelial cells (11, 12). These include members of the epithelial growth factor/transforming growth factor and the fibroblast growth factor families (11) as well as keratinocyte growth factor (32) and IL-1, IL-2, IL-4, IL-15, and IFN-γ (11). For example, IL-15, produced by activated macrophages (33), can up-regulate the expression of transforming growth factor-β, which in turn can stimulate epithelial cell migration and thereby promote recovery of epithelial integrity after wounding (34). JBT 3002 is a potent activator of macrophages (23, 24), and its oral administration can activate tissue macrophages to generate numerous cytokines that affect proliferation and turnover of intestinal epithelial cells (19, 22, 23). We are currently attempting to identify these specific cytokines.

Mucosal infections due to translocation of aerobic bacteria may be another mechanism responsible for chemotherapy-induced diarrhea (10). Studies from several laboratories have suggested that macrophage-derived cytokines are of particular importance for the preservation of mucosal immunity through activation of lymphocytes within the lamina propria. Cytokines may regulate mucosal immunity; these include: (a) IL-6, a differentiation factor for B cells that can induce production of secretory IgA, which protects the mucosal surface area from pathogens (13, 14); (b) IL-15, a regulator of proliferation and differentiation of T cells (15) and B cells (16); and (c) IL-18, an IFN-γ-inducing factor (17). Our results show that changes in intestinal immune cell populations produced by treatment with irinotecan are characterized by the loss of lymphocytes and a diffuse infiltration of macrophages in response to mucosal inflammation. Combination therapy with JBT 3002 and irinotecan, however, preserved the cellular composition within the lamina propria. JBT 3002 has been shown to induce production of macrophage-derived cytokines including IL-1 and IL-6 (24); and, therefore, systemic administration of JBT 3002 may enable intestinal macrophages to release cytokines that activate the lymphocytes within the lamina propria, which would result in the preservation of mucosal immunity during chemotherapy.

It is well established that systemic treatment of mice with liposomes that contain immunomodulators activates macrophages to a tumoricidal state and can cause eradication of lymph node, lung, and liver metastases (22). However, the efficacy of this form of immunotherapy is limited to minimal tumor burden associated with micrometastases or residual tumor after conventional therapy (35–37). The results presented here demonstrate the efficacy of combining chemotherapy with biological therapy. Although treatment of mice with irinotecan once a week did result in a significant reduction of liver metastasis, the addition of orally administered JBT 3002 led to superior therapeutic results, most likely because of the tumoricidal properties of inflammatory macrophages. Blood monocytes migrate to areas of inflammation (38), and the induction of inflammatory changes by local thoracic irradiation has been shown to produce significant regression of experimental fibrosarcoma pulmonary metastases in mice treated with systemic injections of liposomes containing macrophage activators (21). Inflammation and necrotic tissues (39) were observed in the mice treated with irinotecan. Notably, combination therapy with JBT 3002 and irinotecan (but not JBT 3002 alone) led to enhanced infiltration.
Prevention of Mucositis by Oral JBT 3002

of macrophages within the liver metastases, suggesting that inflammatory changes due to irinotecan treatment are chemotactic to activated macrophages. The enhanced antitumor effects observed in mice that were given both irinotecan and JBT 3002 may also have been due to the increased sensitivity of target CT-26 tumor cells to the toxic effects of irinotecan in the presence of activated macrophages. In vitro treatment of human ovarian cancer cells with irinotecan plus IL-1a (40) has been shown to produce additive cytotoxicity, and macrophages that are incubated with JBT 3002 release IL-1a (24). Whether IL-1a and irinotecan produce additive antitumor effects against CT-26 cells is currently being investigated.

CPT-11 is metabolized to SN-38 in mouse serum (41) and liver (42) by the enzyme carboxylesterase (42). One distinct possibility for the enhanced effects of CPT-11 in mice that receive oral JBT-3002 may be the increased carboxylesterase activity in macrophages. This possibility is presently under investigation.

In summary, we have demonstrated the ability of orally administered JBT 3002: (a) to prevent irinotecan-induced dose-limiting gastrointestinal toxicity; (b) to maintain lymphocyte and monocyte populations within the lumina propia, a prerequisite for intact mucosal immunity; and (c) to induce potent tumoralnicous properties in tissue macrophages, resulting in an enhanced therapeutic effect of irinotecan. The pleiotropic effects of immunomodulation by oral JBT 3002 against experimental liver metastases described here, together with our previous observations that the monocypotaxia and intestinal damage caused by doxorubicin or X-irradiation can be prevented by immunomodulation with MTP-PE (19), recommend the clinical use of orally administered JBT 3002 combined with irinotecan for the treatment of advanced colon cancer.

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